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Elevated levels of multiple cytochrome P450 forms in tilapia from Billings Reservoir-São Paulo, Brazil

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Abstract

Cytochrome P4501A (CYP1A) levels in tissues of fish inhabiting polluted areas have been used extensively in biomonitoring studies in Europe and North America. However, little information is available about the extent of CYP1A expression in fish from South American waters, nor on the expression of other CYP proteins in fish from polluted sites. The content of total cytochrome P450 and b_5 and of proteins cross-reactive with antibodies to teleost (scup) CYP1A-, CYP2B- and CYP3A-like proteins, as well as rates of alkoxy-resorufin-*O*-dealkylases (ARODs) with methoxy-, ethoxy-, pentoxy- and benzyloxyresorufin substrates, were analyzed in liver and kidney microsomes from tilapia (*Oreochromis niloticus*) from a reference site (Estação de Piscicultura de Pindamonhangaba, São Paulo) and a polluted site (Billings Reservoir, São Paulo, Brazil). Levels of total microsomal P450 and b_5 were elevated 1.8- and 3.3-fold, respectively, in liver and 4.3- and 2.4-fold in kidney of tilapia from Billings Reservoir. Rates of all four ARODs and the levels of CYP1A-, CYP2B- and CYP3A-cross-reactive proteins were elevated in liver and kidney of fish from Billings Reservoir compared to those in reference fish. Hepatic microsomal EROD and MROD were about 20-fold greater and levels of 1A protein more than 400-fold greater in Billings fish. Antibodies to teleost CYP1A and putative CYP3A recognized single bands while Pab to the scup CYP2B-like protein recognized three protein bands in liver and kidney. This suggests that there are multiple CYP2B-like proteins expressed in tilapia. Immunohistochemical analysis also indicated increased expression of CYP1A cross-reactive proteins in cells of liver, gill, kidney and heart. The apparent induction of CYP1A probably reflects the exposure to aryl hydrocarbon receptor agonists present as pollutants in the Billings Reservoir. Elevated levels of the other CYP forms in tissues of tilapia from Billings Reservoir could be related either to the presence of pollutants or to some natural compound present in the diet, since tilapia is known as an herbivorous species.

Keywords: Cytochrome P450; Tilapia; Billings reservoir; CYP forms; ARODs

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1. Introduction

Measurements of biological changes induced by chemical contamination in aquatic environments may be useful in biomonitoring programs for detecting exposure to those chemicals (Stegeman et al., 1992). This approach has been used extensively in aquatic ecosystems in North America and Europe (Stegeman, 1993; Bourgeot et al., 1996). However, studies of biological responses induced by pollutants in South American waters are scarce. In Brazil, there are some regions, such as in the city of São Paulo, where the environmental problems are extreme. São Paulo, the largest city in South America has an estimated population of more than 15 million people and about 1250 industries (Carvalho et al., 1998). During the last decades, many cases of contamination by heavy metals and organic compounds, such as PCBs, organochlorine pesticides, polycyclic aromatic hydrocarbons have been observed in waters in and around São Paulo, especially in the Billings Reservoir (Fig. 1). This Reservoir receives periodic discharges of domestic and industrial effluents that have occasionally caused a high mortality of tilapia, one of the most abundant fish in this area (Costa et al., 1998). Since tilapia is widely distributed around the world and appears to be highly resistant to the presence of pollutants, some authors have suggested tilapia as indicator species for effects of environmental pollution in tropical regions (Ueng and Ueng, 1995).

Recently, Bainy et al. (1996) observed an unusually high level of total microsomal cytochrome P450 in liver and kidney of tilapia caught at Billings Reservoir (Fig. 1). These high levels were associated with the PCBs detected in these organisms, and may also have been related to oxidative stress observed in several tissues of these animals (Bainy et al., 1996).

The cytochrome P450 (CYP) system plays a pivotal role in metabolism of xenobiotics and its function can be related to species differences in susceptibility to the toxic effects of foreign chemicals. The most extensively studied CYP forms in fish belong to the CYP1A subfamily. These proteins are widely used as biomarkers of aquatic contamination, since they are induced by toxic compounds, most notably polynuclear aromatic hydrocarbons (PAHs), planar polychlorinated biphenyls congeners (PCBs), 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and 2,3,7,8-tetrachlorodibenzofuran (TCDF) (Stegeman, 1993; Rattner et al., 1993; Bucheli and Fent, 1995). Little information is available about the effects of environmental chemicals on other CYP forms in fish. Celander et al. (1996a) reported that in trout liver, the expression of proteins cross-reactive with antibodies to CYP2B- and CYP3A-like proteins were not affected by PCB treatment. The induction of CYP2B in mammals is associated with phenobarbital (PB), but this effect is not observed in fish (Kleinow et al., 1987). There are apparent 2B homologues in fish, but these may have a different regulatory mechanism from that controlling expression of these genes in mammals (Stegeman, 1993). CYP3As in mammals catalyze steroid 6β-hydroxylase, and are induced by and metabolize glucocorticoids and natural products (Schuetz et al., 1984). Putative CYP3A proteins have been purified and CYP3A sequences cloned (Celander and Stegeman, 1997) from various fish, but important questions still remain to be addressed concerning the structure, physiological function and regulation of teleost CYP3As (Celander et al., 1996b).

The purpose of this study was to analyze the expression of CYP1A-, CYP2B- and CYP3A-like proteins and the activity of some alkoxy-resorufin-*O*-dealkylases (ARODs) in both liver and kidney of Nile tilapia (*Oreochromis niloticus*) collected at a polluted site (Billings Reservoir) and at a reference site (Fish Farm). Ethoxyresorufin-*O*-deethylase (EROD) is primarily a catalytic function of CYP1A, but which P450s catalyze other ARODs in fish has not been established. Complementary immunohistochemical analysis for CYP1A was done in gill, heart, liver and kidney of tilapia from both sites in order to identify the cellular sites of expression of this protein in this species. The results show a high level induction of CYP1A, and possibly effects on CYP2B and CYP3A-like proteins at the Billings Reservoir site.

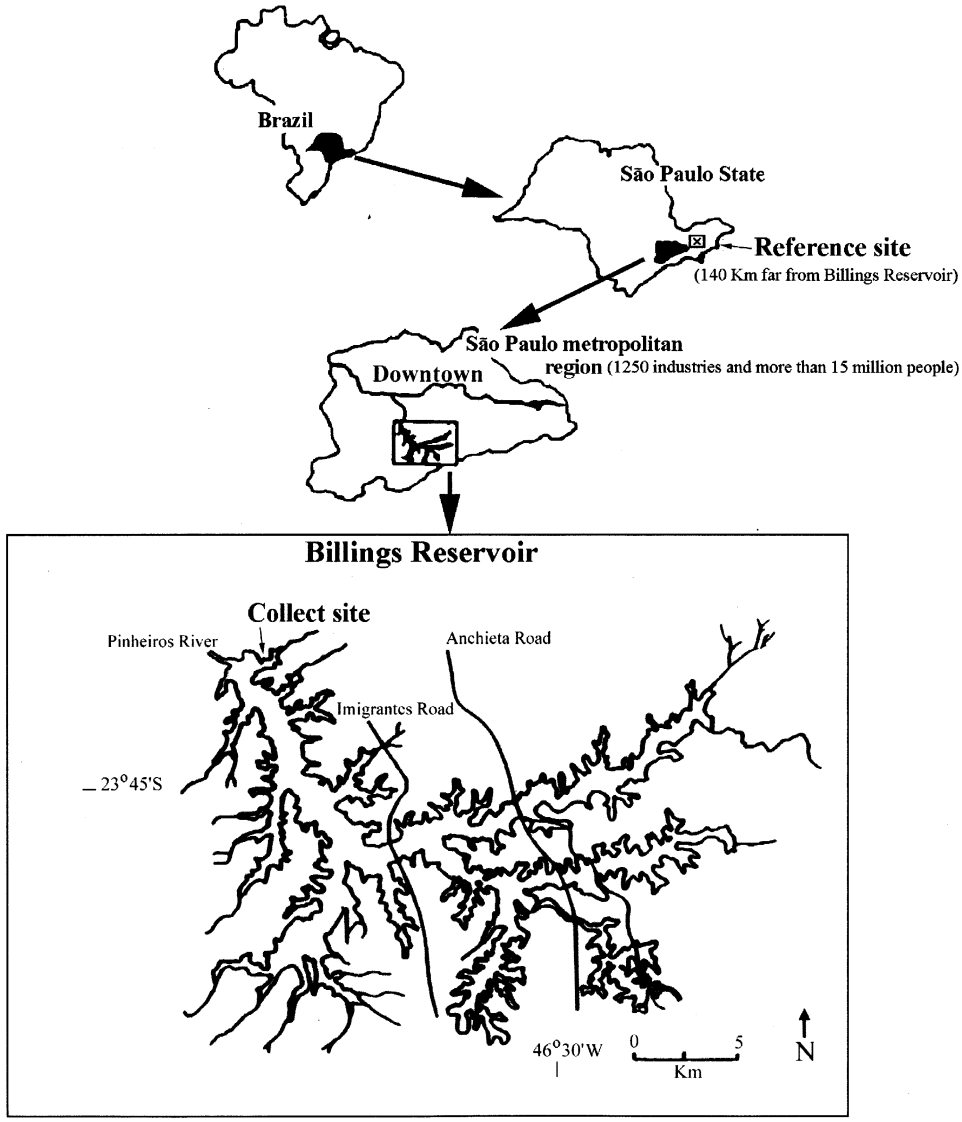

Fig. 1. Map of Billings Reservoir and sampling sites, São Paulo, Brazil.

2. Materials and methods

2.1. Chemicals

The 7-ethoxyresorufin, 7-penthoxyresorufin, 7-methoxyresorufin and 7-benzyloxyresorufin were purchased from Molecular Probes (Eugene OR). Nicotinamide adenine dinucleotide phosphate (reduced form; NADPH), dithiothreitol (DTT), TRIS and buffer salts were obtained from Sigma and glycerol was obtained from Fisher Scientific.

2.2. Fish collection

In November 1995, Nile tilapia (*O. niloticus*) were collected both at a polluted site of the Billings Reservoir (Fig. 1) and at a reference site at a fish farm (Estação de Piscicultura de Pinda-

monhangaba, Instituto de Pesca do Estado de São Paulo) by using cast nets. The water temperature at these sites was 23 and 21°C, respectively. Fish were transported to the laboratory of Dr Virgínia B.C. Junqueira, at the Departamento de Bioquímica, Instituto de Química, Universidade de São Paulo and immediately killed by cervical section. Male and female fish were collected from both sites. The length and weight (mean $\pm$ S.D.) of the fish caught in both sites were 17.9 ± 2.7 cm and 205.4 ± 77.9 g ($n = 13$), and 17.3 ± 2.3 cm and 189.3 ± 74.4 g ($n = 14$), respectively for the reference group and the Billings group. None of the fish used were sexually mature. The reference fish were fed with Purina fish chow. These animals had not been treated with antibiotics, hormones or other food additives.

Sections of gill, liver, heart and kidney were removed and immediately fixed in 10% neutral buffered formalin (pH 7.4) for immunohistochemical analysis (see below). The remaining portions of liver and kidney were immediately frozen in liquid nitrogen and transported in dry-ice to Woods Hole, MA, where microsome preparation and analysis were done.

2.3. Microsome preparation

Frozen tissues were thawed on ice and homogenized in 4 vols. of homogenization buffer (50 mM Tris, 0.15 M KCl, pH 7.4), and microsomal fractions were prepared and resuspended as before (Stegeman et al., 1979). The microsomal protein content was measured using the bicinchoninic acid assay (Smith et al., 1985), with bovine serum albumin (BSA) as a standard.

2.4. Microsomal assays and immunoblotting

Cytochrome b_5 and total cytochrome P450 were determined spectrally as described previously (Stegeman et al., 1979). Microsomal ethoxyresorufin *O*-deethylase (EROD) activity was determined in duplicate, using the fluorimetric method of Hahn et al. (1993), with a Cytofluor 2300 (Millipore) multiwell plate reader; substrate concentration in the assay was 2 μM. The same method was used to analyze the activity of pentoxyresorufin *O*-dealkylase (PROD), methoxyresorufin *O*-demethylase (MROD) and benzyloxyresorufin *O*-dealkylase, but substituting these substrates for ethoxyresorufin, each at concentration of 5 μM in the enzyme assay.

Immunoblot analysis of the microsome preparations was performed as described by Kloepper-Sams et al. (1987). Microsomal proteins (30 to 80 μg lane^{-1}) were resolved on 12% SDS-PAGE gels and then electrophoretically transferred to 0.2 μm nylon sheets (Schleicher and Schuell) and blocked with 1% Schleicher and Schuell blocking powder (w/v) dissolved in Tris-buffered saline, pH 7.4 (blocking solution). Prior to blocking, the proteins were visualized using the reversible protein stain Ponceau S. The primary antibodies used were monoclonal antibody 1-12-3 against scup P450E (CYP1A) (10 μg ml^{-1} blocking solution) (Park et al., 1986; Morrison et al., 1995); rabbit polyclonal antibody against scup CYP2B-like protein (formerly P450B) (10μg ml^{-1} blocking solution) (Klotz et al., 1986) for CYP2B-like protein detection; and rabbit polyclonal antibody against rainbow trout P450con (sera diluted 1:5000 in blocking solution) (Celander et al., 1989) for CYP3A-like protein detection. The secondary antibodies used were either alkaline phosphatase-(AP) conjugated goat anti-rabbit IgG or AP-conjugated goat anti-mouse IgG diluted 1:10000 in blocking solution. The protein bands were detected according to the Schleicher and Schuell Rad-free Kit for Chemiluminescence detection of Western blots and the protein bands were visualized by autoradiography on Fuji Medical X-ray film. Development times for autoradiography were varied depending on the antibody used, to optimize detection of positive cross-reactions within the linear response range of the detection system. The autoradiograms were subsequently photographed with a Kodak DCS200 digital camera and densitometrically analyzed using NIH image. Content of the CYP1A in the microsome preparations was quantified by comparison to BNF-treated scup standards of known CYP1A concentration. Relative content of CYP2B- or CYP3A-like proteins was determined using video image analysis and by com-

Table 1
Levels of total CYP, CYP forms, cytochrome b_5 and associated monooxygenases in liver microsomes of Nile tilapia from Billings and reference site

Parameter	Reference site	Billings reservoir	Billings/reference ratio
Total cytochrome P450 (ηmol mg^{-1} protein)	0.46 ± 0.13 (7)	0.81 ± 0.22 (6)	1.8*
Cytochrome b_5 (ηmol mg^{-1} protein)	0.06 ± 0.01 (7)	0.20 ± 0.06 (7)	3.3*
EROD (ηmol min^{-1} mg^{-1} protein)	8.9 ± 4.4 (12)	203.2 ± 137.7 (13)	22.8*
MROD (ηmol min^{-1} mg^{-1} protein)	2.0 ± 2.7 (10)	39.7 ± 28.8 (11)	19.9*
PROD (ηmol min^{-1} mg^{-1} protein)	1.1 ± 1.2 (10)	6.2 ± 3.4 (11)	5.6*
BROD (ηmol min^{-1} mg^{-1} protein)	1.7 ± 0.6 (9)	5.3 ± 1.8 (9)	3.1*
CYP1A-like protein (ρmol P450E equivalents μg^{-1} protein)	0.18 ± 0.14 (12)	85.9 ± 31.2 (13)	477.2*
CYP2B-like protein (integrated density μg^{-1} protein)	0.37 ± 0.17 (12)	0.98 ± 0.17 (13)	2.6*
CYP3A-like protein (integrated density μg^{-1} protein)	0.19 ± 0.11 (12)	0.83 ± 0.15 (13)	4.4*

All values represent mean ± S.D. Number between parentheses represent sample size.
* Indicates significant differences by Student t-test for $P = 0.001$.

parison of the integrated density per μg of cross-reactive proteins in each sample.

2.5. Immunohistochemical staining

Fixed sections of liver, gill, heart and kidney were embedded in paraffin using standard methods (Luna, 1968). Serial sections (5 μm) of tissue were mounted on Superfrost Plus slides, and stained as previously described by Smolowitz et al. (1991). Prior to staining, the sections were deparaffinated and hydrated. While being hydrated, the sections were incubated in 3% hydrogen peroxide (v/v) to block the activity of endogenous peroxidases. The hydrated sections were stained using an indirect peroxidase stain (universal immunoperoxidase staining kit, Murine, Signet Lab, Dedham, MA). The primary antibody was MAb 1-12-3 or non-specific mouse myeloma IgG (Organteknicon) and secondary antibody was a peroxidase-labeled goat anti-mouse IgG. The degree and intensity of antibody staining in the slides were analyzed according to Smolowitz et al. (1991) and Woodin et al. (1997).

2.6. Statistical analysis

All values are given as means ± S.D. Statistical analysis was performed with StatView™ for the Macintosh, using Student t-test for unpaired data.

3. Results

3.1. Total CYP, cytochrome b_5 and AROD activities in liver and kidney microsomes

Total spectrally-determined P450 levels were markedly higher in both liver (1.8-fold) and kidney (4.3-fold) microsomes of tilapia from Billings Reservoir, compared with the reference fish (Tables 1 and 2). Likewise, the b_5 levels were elevated 3.3-fold in liver and 2.4-fold in the kidneys of fish from the polluted site. The total P450 levels were significantly higher in the liver than in kidney from fish within a site, but the b_5 levels were similar in the two tissues (Tables 1 and 2).

The rates of the hepatic AROD activities with all four substrates were significantly elevated in the Billings fish compared to the reference fish (Table 1). The EROD rate was 22.8-fold higher in Billings than in the reference fish, followed by MROD (19.9-fold), PROD (5.6-fold) and BROD (3.1-fold) (Table 1). In the kidney microsomes, EROD and MROD showed significantly elevated rates (5.5-fold and 1.6-fold, respectively) in Billings fish, compared with the reference group, but no significant differences were observed for BROD and PROD activities (Table 2).

3.2. Immunoblot for CYP1A-, CYP2B- and CYP3A-like proteins in liver and kidney

Results of immunoblot (Western blot) analysis

Table 2
Levels of total CYP, CYP forms, cytochrome b_5 and associated monooxygenases in kidney microsomes of Nile tilapia from Billings and reference site

Parameter	Reference site	Billings reservoir	Billings/reference ratio
Total cytochrome P450 (ηmol mg^{-1} protein)	0.09 ± 0.04 (6)	0.39 ± 0.15 (6)	4.3*
Cytochrome b_5 (ηmol mg^{-1} protein)	0.09 ± 0.02 (6)	0.22 ± 0.15 (6)	2.4*
EROD (ηmol min^{-1} mg^{-1} protein)	2.8 ± 1.3 (12)	15.5 ± 7.0 (12)	5.5*
MROD (ηmol min^{-1} mg^{-1} protein)	4.7 ± 2.0 (12)	7.3 ± 3.2 (12)	1.6*
PROD (ηmol min^{-1} mg^{-1} protein)	3.2 ± 1.7 (6)	3.7 ± 1.8 (6)	1.2
BROD (ηmol min^{-1} mg^{-1} protein)	1.1 ± 0.6 (12)	1.5 ± 0.5 (12)	1.3
CYP1A-like protein (ρmol P450E equivalents μg^{-1} protein)	<0.02 (12)	0.034 ± 0.010 (12)	1.7*[a]
CYP2B-like protein (integrated density μg^{-1} protein)	0.31 ± 0.12 (12)	0.47 ± 0.12 (12)	1.5**
CYP3A-like protein (integrated density μg^{-1} protein)	0.07 ± 0.03 (12)	0.10 ± 0.05 (12)	1.4

All values represent mean $\pm$ S.D. Number between parentheses represent sample size.
[a] Ratio calculated by the limit of detection of this methodology.
* Indicates significant differences by Student t-test for $P = 0.001$.
** P 0.05.

of CYP1A-, CYP2B- and CYP3A-like proteins in liver microsomes of tilapia from reference and Billings sites are shown in Fig. 2. The contents of proteins cross-reactive with antibodies to each of the three CYP forms were significantly greater in liver of Billings fish. The most pronounced difference (in scup CYP1A equivalents or in densitometric units) was observed in the CYP1A-cross-reactive form (477-fold). The content of CYP3A-like protein was 4.4-fold and CYP2B-like protein 2.6-fold greater in Billings fish (Table 1).

The immunoblot analysis of kidney microsomes also showed elevated content of CYP1A in Billings fish compared to the content in reference site fish (Fig. 3 and Table 2). Significantly higher levels of CYP2B-like proteins were also visualised in the kidney of Billings fish than of reference fish (Fig. 3 and Table 2). No differences were observed in the CYP3A-like protein levels, between the two groups (Table 2).

Immunoblots with MAb 1-12-3 to scup CYP1A and the PAb to trout P450con each showed, respectively, a single cross-reacting protein band in liver and kidney of tilapia from both sites (Figs. 2 and 3). However, at least three cross-reactive proteins were observed when the liver and kidney blots were incubated with PAb to scup CYP2B-like protein (Fig. 4). The expression of these three bands varied for each fish. A most intense intermediary band (53.9 kDa) was observed in all samples, but a strongly staining faster band (52.3 kDa) and a faintly staining slower migrating band (55.5 kDa) were expressed differentially. Similar patterns were seen in liver and kidney.

3.3. Immunohistochemical localization of CYP1A

3.3.1. Liver

Strong CYP1A-specific staining was seen in hepatocytes and endothelial cells of hepatic arterioles and portal veins of fish from Billings Reservoir (Table 3). In some Billings fish, an intense macrophage aggregation was observed associated with pancreatic acinar cells (Fig. 5). These macrophage aggregates did not stain with 1-12-3. No staining was observed in the liver of reference fish.

3.3.2. Kidney

Tilapia from the Billings Reservoir had moderate to strong CYP1A staining in renal tubular epithelium and endothelial cells of the glomerulae and vessels (Table 3). A very mild CYP1A tubular epithelium staining was observed in three of six reference fish.

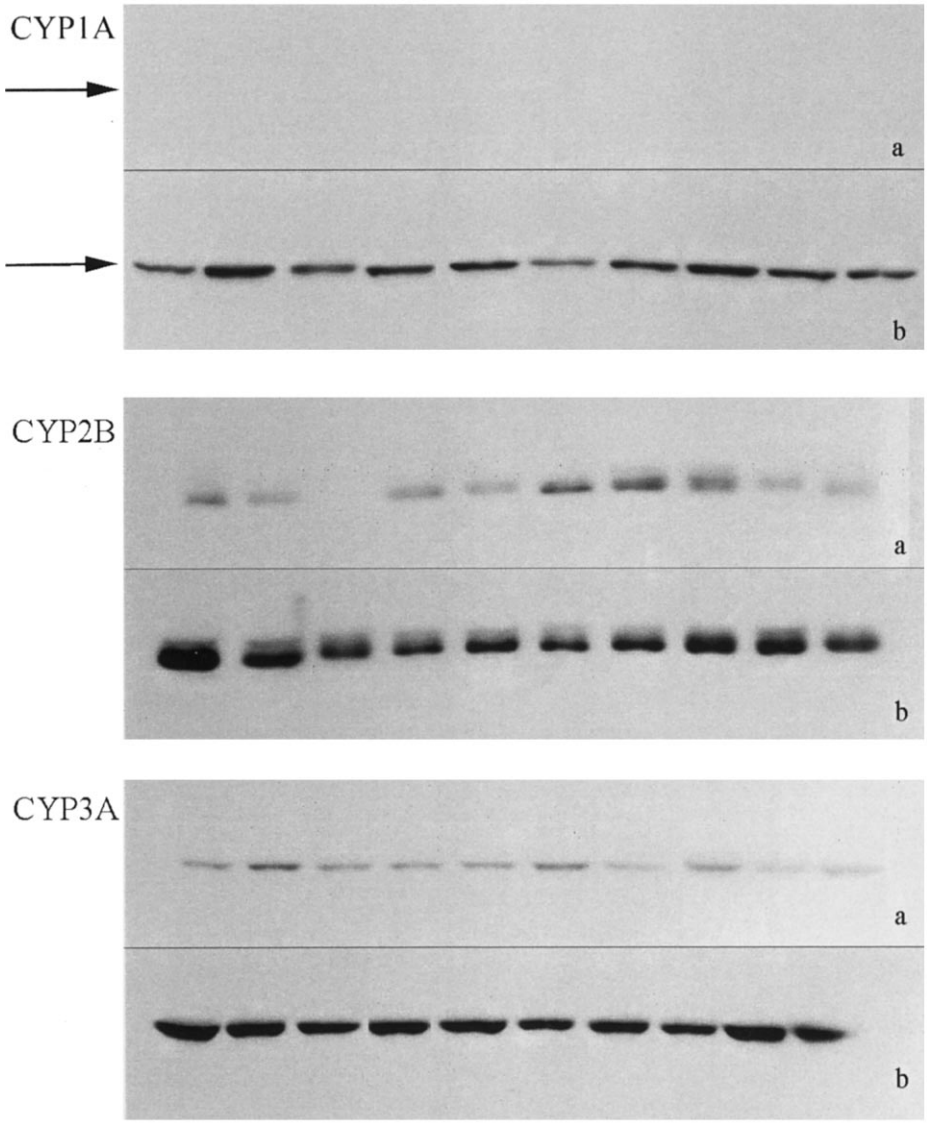

Fig. 2. Western blot analysis of liver microsomes of Nile tilapia from reference and Billings sites. Amounts of protein applied were 20 μg lane^{-1} for CYP1A and 50 μg for CYP2B- and CYP3A-like protein. Blots were stained with MAb 1-12-3 scup P450E, PAb to scup P450B and PAb to trout P450com and secondary antibodies as in Section 2. The panel shows the expression of CYP forms of fish from reference (a) and Billings (b) sites. The CYP forms are indicated. Each lane represents one fish. Arrows identify the position of electrophoretic migration of the control, scup CYP1A, in the gels from reference and Billings fish.

3.3.3. Gill

Very strong positive CYP1A staining was seen in the pillar (endothelial) cells, as well as in gill arch endothelium of Billings fish (Table 3 and Fig. 6). In some cases, moderate CYP1A staining was observed in the gill epithelial cells (Table 3). Gills of reference fish did not stain.

3.3.4. Heart

Endocardial cells of the atrium and ventricule stained moderately and pervasively for CYP1A in heart of Billings fish (Table 3 and Fig. 7). A very mild CYP1A staining was visualized in endocardial cells of the reference fish, but the occurrence of this was rare. Myocytes did not show staining.

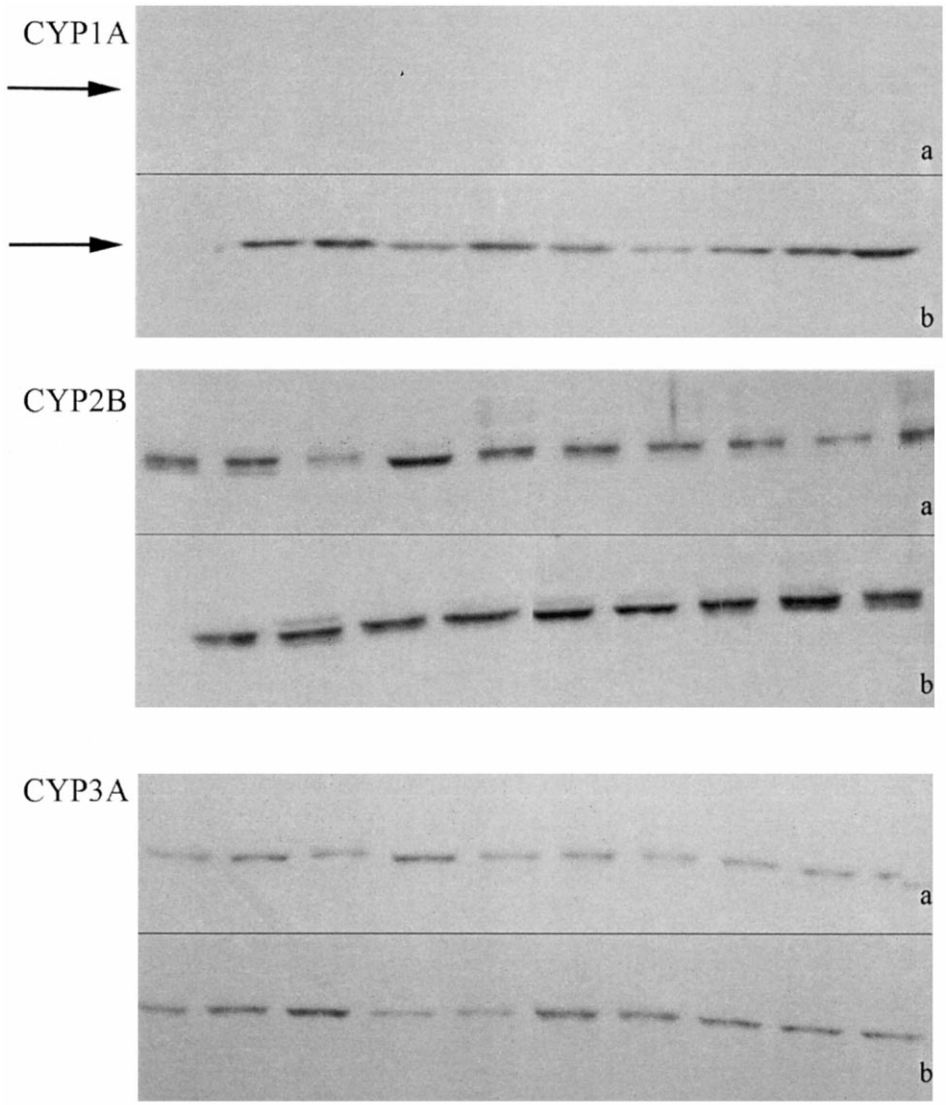

Fig. 3. Western blot analysis of kidney microsomes of Nile tilapia from reference and Billings sites. Amounts of protein applied were 80 μg lane^{-1} for CYP1A and 50 μg lane^{-1} for CYP2B- and CYP3A-like protein. Blots were stained with MAb 1-12-3 scup P450E, PAb to scup P450B and PAb to trout P450con. Goat anti-mouse or goat anti-rabbit AP-conjugated were used as secondary antibodies. The panel shows the expression of CYP forms of fish from reference (a) and Billings (b) sites. The CYP forms are indicated. Each lane represents one fish. Arrows identify the position of electrophoretic migration of the control, scup CYP1A, in the gels from reference and Billings fish.

4. Discussion

4.1. Induction of CYP isoforms in liver and kidney of tilapia

CYP1A induction, detected by immunoassay or by catalytic activity (e.g. EROD) has been widely used as a biomarker of contamination by organic compounds, such as PAH, TCDD/F and coplanar PCBs (Varanasi et al., 1986; Stegeman et al., 1987; Haux and Förlin, 1988; Renton and Addison, 1992; Stegeman et al., 1992). However, there still is little information on the expression of other CYP forms or activities in fish from pol-

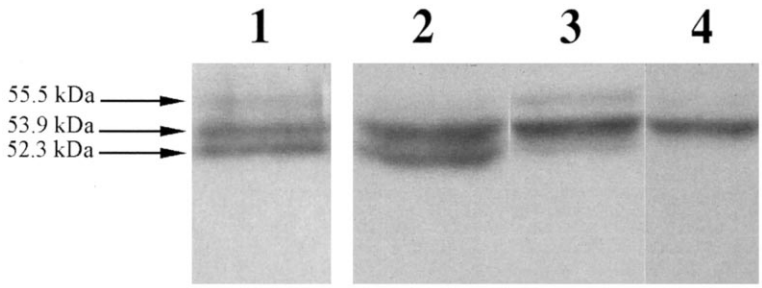

Fig. 4. CYP2B-cross reactive proteins in kidney and liver of Nile tilapia from polluted sites. Immunoblot was developed with polyclonal antibody to scup P450B as the primary antibody. A total of 50 μg of protein were loaded in each lane. 1, Liver sample. 2, 3, 4, Kidney samples. The molecular weights of the bands are indicated.

luted aquatic environments and little information concerning possible CYP1A induction in some regions of the world, notably South America, Africa and Asia (Ueng and Ueng, 1995; Gadagbui et al., 1996). This is the first such study with fish from Brazil. The results showed elevated AROD activities associated with a strong apparent induction of CYP1A, and an elevation of multiple CYP forms in liver and kidney of tilapia caught at the Billings Reservoir site in São Paulo. Likewise, immunohistochemical studies showed elevated CYP1A expression in different tissues of these animals.

Table 3
CYP1A staining in tissues of Nile tilapia from reference site and Billings reservoir

Tissue	Reference site Occurrence[a] × intensity[b] (n/n)[c]	Billings reservoir Occurrence × intensity (n/n)
Liver		
Hepatocytes	0 (0/6)	11.5 ± 1.1 (6/6)
Endothelium	0 (0/6)	8.0 ± 1.2 (6/6)
Heart		
Atrium Endothelium	1 (1/2)	9.7 ± 3.9 (3/3)
Ventricular Endothelium	0.2 ± 0.4 (1/6)	6.2 ± 3.5 (6/6)
Kidney		
Tubules	0.8 ± 0.9 (3/6)	8.1 ± 0.7 (5/5)
Endothelium	0 (0/6)	12.7 ± 4.1 (5/5)
Gills		
Pillar cells	0 (0/5)	14.1 ± 1.2 (5/5)
Lamelar epithelium	0 (0/5)	6.0 ± 2.7 (4/5)
Archae endothelium	0 (0/3)	12.6 ± 5.2 (5/5)

[a] Occurrence was classified according to the scale (0) no staining, (1) rare cells staining, (2) many cells staining, (3) all cells staining.
[b] Intensity of the stain was classified as (0) no staining, (1) very mild, (2) mild, (3) moderate, (4) strong, (5) very strong. The results are expressed as mean ± S.D.; six fish per group were used for this analysis. Some slides did not showed all tissues.
[c] Number of fish positive/number examined.

According to the São Paulo State Environmental Agency (Cetesb, 1993), fish from Billings Reservoir are chronically exposed to high levels of compounds, including PCBs, lindane, hexachlorobenzene, pentachlorophenol and DDE, some of which are common inducers of CYP1A. Previous studies done by Bainy et al. (1996) showed evidence of oxidative stress in various tissues of fish from Billings Reservoir. The cause of that stress is not known, but could involve the elevated levels of various CYP isoforms in those fish (Bainy et al., 1996).

In the present study, we observed levels of total microsomal CYP 1.8-fold higher in liver of Billings fish, compared with the reference fish (Table 1). Immunoblot analysis of these samples showed elevated levels not only of CYP1A-, but also of CYP2B- and CYP3A-cross-reactive proteins, all of which probably contribute to the increase in total P450. Expression of the CYP2B- and CYP3A-reactive proteins in reference fish could be constitutive. The markedly elevated levels of CYP2B and CYP3A in Billings fish is an unusual finding and possibly reflects on induction of these proteins.

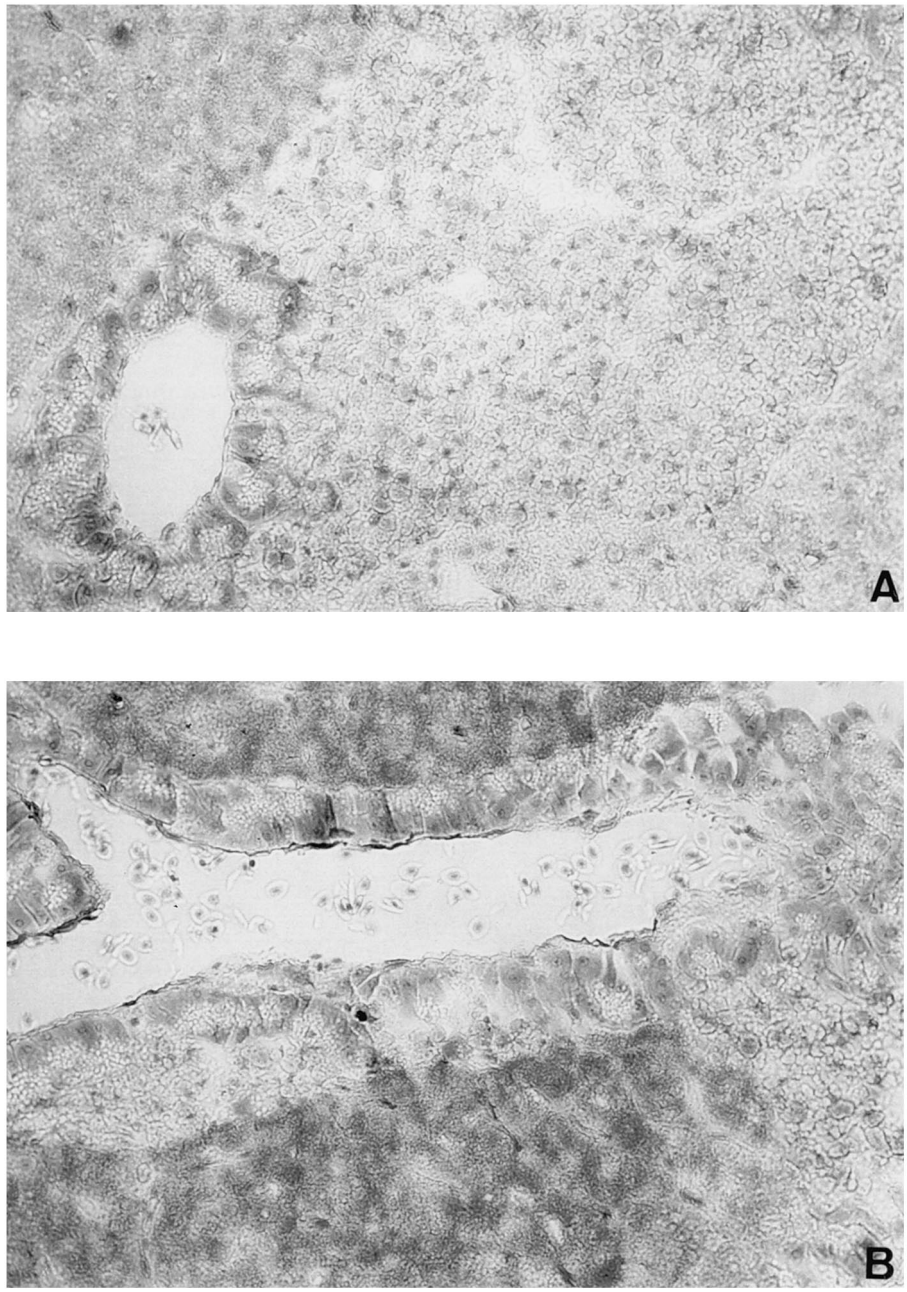

Fig. 5. Immunohistochemical analysis of CYP1A in liver of Nile tilapia from Billings Reservoir. (A) An intense macrophage aggregation was associated with pancreatic cells and hepatocytes (400 ×). (B) Section showing a strong CYP1A specific staining in hepatocytes and endothelial cells (× 400).

In mammals, CYP2B proteins are induced by phenobarbital, ortho-substituted PCB congeners and insecticides (Stegeman and Hahn, 1994). Chemical induction of CYP2B-like proteins in fish has not been identified. The high levels of CYP2B-cross reactive proteins observed in liver

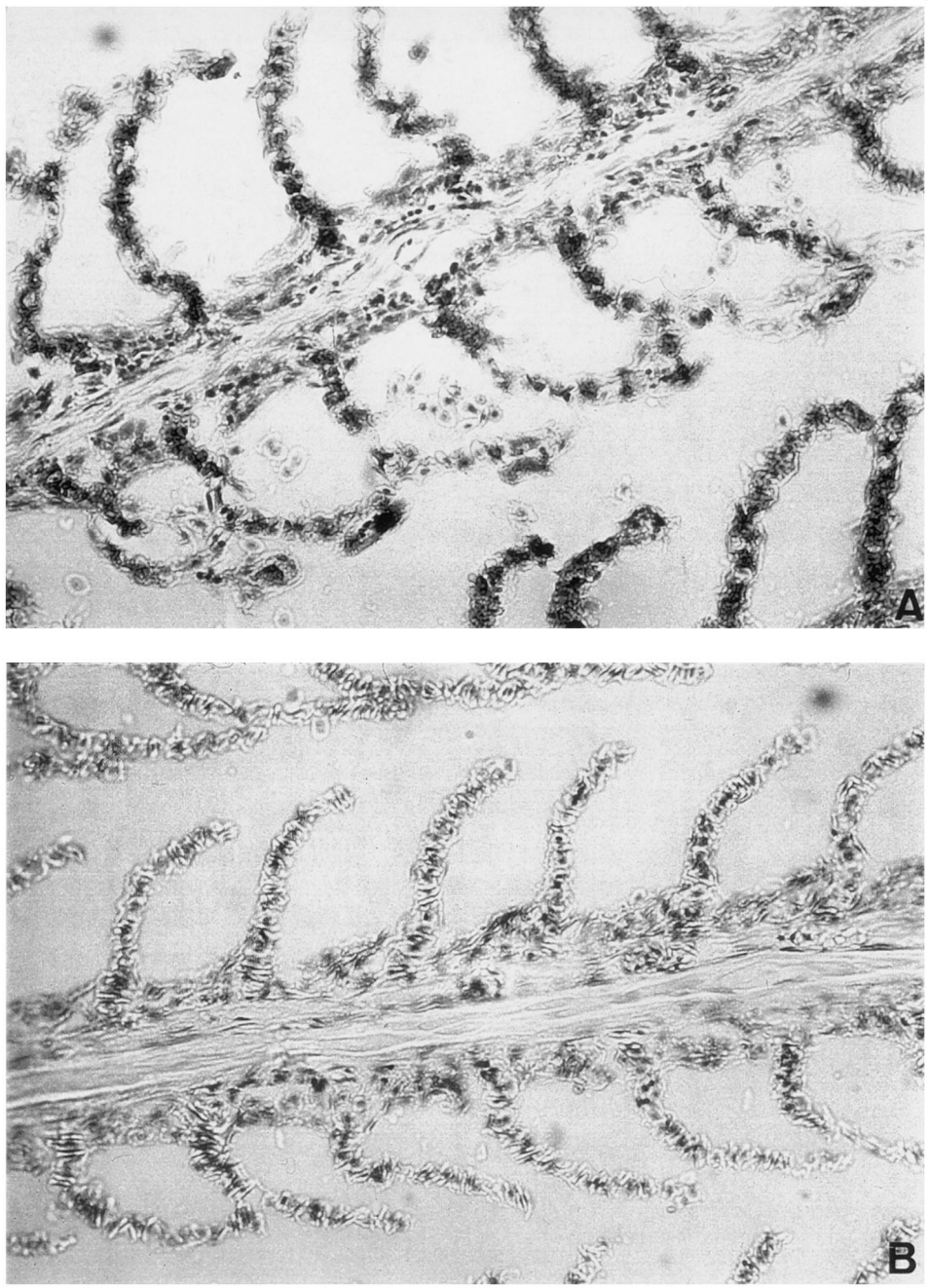

Fig. 6. Immunohistochemical analysis of CYP1A in gill of Nile tilapia from Billings Reservoir (A) and reference site (B). Section A shows a very strong positive CYP1A staining in the pillar (endothelial) and epithelial cells (× 400).

and kidney of tilapia from Billings Reservoir could be caused by some pollutants. Total PCB levels of 681 μg kg^{-1}, lindane levels of 2 μg kg^{-1}, DDE levels of 5 μg kg^{-1} were detected in the sediment of Billings reservoir (Lamparelli et al., 1996). Recent analysis done in the sediment

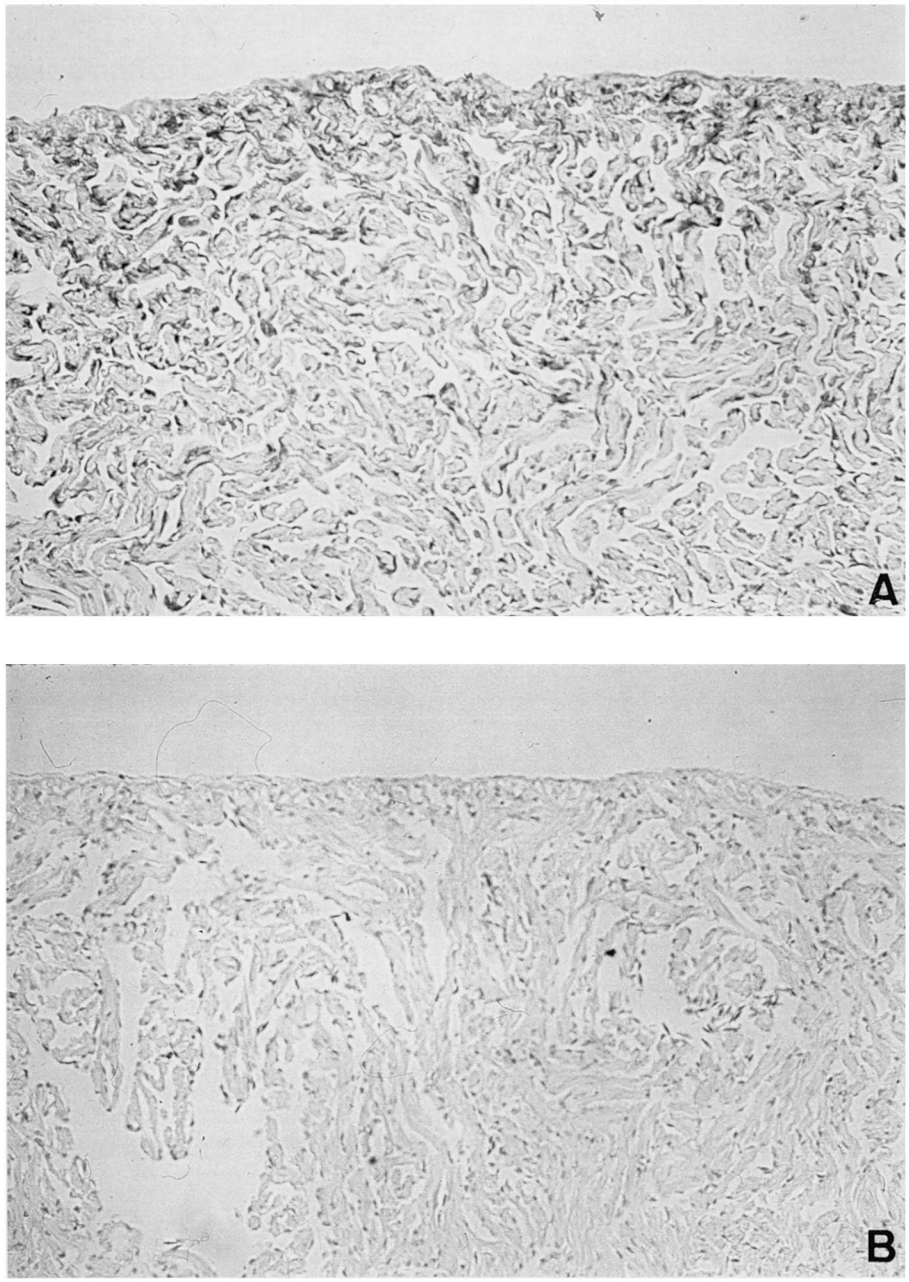

Fig. 7. Immunohistochemical analysis of CYP1A in heart of Nile tilapia from Billings Reservoir (A) and reference site (B). Section A shows a moderate and pervasive CYP1A staining in the atrium endocardial cells (× 400).

showed an average hexachlorobenzene levels of 0.87 μg kg^{-1}, DDE levels of 23.3 μg kg^{-1}, and DDT levels of 15.5 μg kg^{-1} (Costa et al., unpublished data). Table 4 shows the levels of total PCBs, BHC, lindane, 4,4′ DDE and HCB detected by Costa et al. (1998) and Araújo (1998) in muscle and viscera of tilapia collected at Billings reservoir. Whether any of these chemicals might

Table 4
Levels of total PCBs, BHC, Lindane, 4,4′ DDE and HCB in axial muscle and viscera of tilapia collected at Billings reservoir

	Axial muscle[a]				Viscera[b]			
	Minimum	Maximum	Average	*n*	Minimum	Maximum	Average	*n*
Total PCBs ($\mu g\ kg^{-1}$)	ND[c]	101.4	15.46	20	13.2	313.0	98.0	5
BHC ($\mu g\ kg^{-1}$)	ND	12.8	2.21	20	ND	27.1	5.9	5
Lindane ($\mu g\ kg^{-1}$)	ND	3.7	1.3	9	ND	8.4	1.9	5
4,4′ DDE ($\mu g\ kg^{-1}$)	ND	ND	ND	9	ND	0.6	0.1	5
HCB ($\mu g\ kg^{-1}$)	ND	6.5	0.9	20	ND	2.9	0.8	5

[a] Costa et al., 1998.
[b] Araújo, 1998.
[c] ND, not detected.

be responsible for CYP2B induction in tilapia remains to be determined.

Enhanced levels of CYP2B in Billings fish might also be due to differences in dietary compounds. Some studies have shown that tilapia is an omnivorous feeder, but considerable evidence exists suggesting that they are primarily herbivorous (Dempster et al., 1993). Stomach content analysis has shown that tilapia from Billings Reservoir feed mainly on algae and algae-based detritus (Carvalho and Costa, unpublished data). The reference fish obtained from Estação de Piscicultura de Pindamonhangaba were fed with commercial fish chow. The possibility that the augmented expression of CYP2B-like proteins in fish may be related to dietary compounds has been considered in studies of other tropical fish. Vrolijk et al. (1994) observed that *Chaetodon capistratus*, a gorgonian-feeders had a greater content of total P450 and proteins recognized by antibodies to scup P450B (putative CYP2B) than the two other simpatric congeneric butterflyfish species, that do not feed on gorgonians. Gorgonians are rich in terpenoids that are known inducers of the CYP2B and CYP3 gene families in mammals (Nebert et al., 1989). Similarly, Stegeman et al. (1997) reported high levels of CYP2B-like proteins in herbivorous fish from Bermuda. However, while the Billings reservoir fish showed evidence of consuming a plant diet, there are no available data concerning the identity of natural products present in that diet.

At least three CYP2B-like proteins were detected in the liver and kidney blots when incubated with PAb to scup CYP2B-like protein (Fig. 4). A more intense intermediary band was observed in all samples, but a faster migrating band and a slower migrating band appeared to be expressed differentially in individual fish. This result may indicate the existence of multiple CYP2B-related genes in tilapia. Similarly, Celander et al. (1996a) observed two protein bands in liver microsomes of rainbow trout incubated with the same antibody and Stegeman et al. (1997) reported multiple P450B cross-reactive proteins in fish from Bermuda. The finding of such multiple 2B-cross reactive proteins in several fish species suggests that CYP2B related proteins may play diverse role in fishes. Further studies are needed to establish the identity and function of these proteins.

Elevated expression of CYP3A-cross reactive proteins was observed in liver of tilapia from Billings Reservoir, but not in the kidney of these fish. Similar to mammals, CYP3As in fish are the major enzymes responsible for 6β-hydroxylation of steroids (Celander et al., 1996b). In humans, CYP3A enzymes are induced by numerous drugs, such as rifampin, dexamethasone, phenobarbital and phenytoin (Pichard et al., 1990). However, very little is known about the CYP3A-like protein inducibility in fish. Celander et al. (1989) observed a slight elevation in the levels of P450con-immunoreactive proteins (putative CYP3A) in juve-

nile rainbow trout treated with cortisol and pregnelone 16α-carbonitrile (PCN). Recently, Pathiratne and George (1996) observed a 2-fold induction of protein detected with anti-P450con (putative CYP3A) in tilapia (*Oreochromis niloticus*) treated with PCN. In contrast, the P450con (3A) protein levels in rainbow trout remained unchanged after prolonged exposure to PCB (Celander et al., 1996a). Vrolijk et al. (1994) suggested that the presence of allelochemicals in the diet could cause induction of CYP3A-related protein content in the marine butterfly fish *Chaetodon capistratus*. Thus, we speculate that the elevated expression of CYP3A-related proteins in liver could be the result of exposure to chemicals in the Billings Reservoir and/or to the presence of allelochemicals in the diet. Whether the chemicals detected in the sediment of Billings Reservoir and in the tilapia muscle and viscera, as shown previously (Lamparelli et al., 1996; Araújo, 1998; Costa et al., 1998) are responsible for inducing CYP3A-related proteins remains to be clarified. Likewise, investigation is needed on the identity of allelochemicals that may occur in the diet of tilapia, and whether these could be responsible for such induction.

4.2. Induction of alkoxyresorufin O-dealkylases in liver and kidney of tilapia

The induction of different CYP forms can be detected through enzymatic assays. Some 7-alkoxyresorufin analogs have been used for monitoring the induction of CYP1A and CYP2B-like enzymes in birds (Rattner et al., 1993). In rat and mouse, CYP1A enzymes preferentially catalyze the *O*-dealkylation of 7-methoxyresorufin and 7-ethoxyresorufin, whereas CYP2B enzymes preferentially catalyze the *O*-dealkylation of 7-pentoxyresorufin and 7-benzyloxyresorufin (Parkinson, 1996). The EROD and MROD are catalyzed by CYP1A in fish as well as in mammals. We observed that rates of all four activities, EROD, MROD, PROD and BROD were greater in liver microsomes from the Billings fish whose levels of CYP1A and CYP2B-related proteins were elevated. Similarly in kidney, CYP1A catalytic activities (EROD and MROD) were elevated, consistent with the elevated levels of CYP1A observed in this tissue. However, despite the slight but significant increase of CYP2B-related proteins in kidney, no site related differences were observed in the activity of renal microsomal BROD and PROD. Whether these enzyme activities are associated with CYP2B in kidney of tilapia remains to be clarified. Recently, a novel fish CYP, CYP2N2 has been found to catalyze BROD and PROD (Oleksiak and Stegeman, unpublished data), but the identity of other teleost CYP that carry out these reactions has not been established.

4.3. Cellular localization of CYP1A

The results showed CYP1A induction in different cell types in liver and some extrahepatic organs of tilapia from the Billings Reservoir. Hepatocytes, renal tubule epithelium, endothelial cells of hepatic arterioles, portal veins, renal glomerulae, endothelial cells in gill arch, endocardial cells of the atrium and ventricule all showed moderate to strong CYP1A-specific stain. Very strong CYP1A staining was seen in the pillar (endothelial) cells in gills.

There is an apparent conflicting result in that in the kidney, where lower CYP1A levels were determined by western blotting, there is a considerable staining of tubule epithelium, compared to that in hepatocytes (Table 3 and Figs. 2 and 3). However, the mass of hepatocytes in the liver is much greater than the mass of endothelium or tubular cells.

The results are similar to patterns of CYP1A induction in fish exposed to PAH experimentally or in the environment (Stegeman et al., 1989, 1991; Lester et al., 1993), and are fully consistent with the fact that Billings fish have been exposed to appreciable levels of CYP1A inducers.

The strong staining of CYP1A in endothelial cells in all organs examined corroborates other studies showing that endothelium is a major site of CYP1A induction. As proposed before by Stegeman et al. (1991), these cells can act as a first barrier to protect different organs against the toxicity of CYP-inducers. However, the induction of CYP1A in endothelium could be involved in the etiology of some diseases in aquatic organisms (Guiney et al., 1997).

In summary, high level of expression of CYP1A in liver and other organs indicates a strong induction, presumably due to exposure of Billings fish to AhR receptor agonists. Consequences to fish in the Reservoir are not clear. In addition, there was apparent increase of CYP2B and CYP3A-related proteins, which suggests that tilapia, like some other tropical species (Stegeman et al., 1997), may be a good model for investigating the regulation of those proteins. Chemical analysis of water, sediment and biota from the Billings Reservoir could indicate the identity of chemicals that can induce these proteins in fish. The results here provide further support for the use of CYP1A in tilapia (Ueng and Ueng, 1995) in evaluating contamination by suspect Ah-receptor agonists in tropical systems.

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